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Fauna and taxonomy of Diamesinae (Diptera, Chironomidae) from the Caucasus, with a morphological description and DNA barcoding of new taxa and a discussion of diagnostic problems for *Diamesa* Meigen and *Pseudodiamesa* Goetghebuer

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Abstract

As a result of the revision of adult males as well as available literature data, 26 species of the subfamily Diamesinae are registered for the Caucasus, belonging to 5 genera. Four species are recorded for the first time for this region, one species, *D. elbrusica* **sp. nov.,** and one subspecies, *D. sakartvella gidanica* **subsp. nov.**, are new to science and are described. Six species are classified as endemics of the Caucasus. Distribution of other species of Caucasian Diamesinae is discussed.

DNA barcodes of 102 specimens and 20 species of four genera, *Boreoheptagyia* Brundin, *Diamesa* Meigen, *Pseudodiamesa* Goetghebuer and *Syndiamesa* Kieffer were obtained in this study. Of these, 12 species were deposed in the GenBank and BOLD systems for the first time. We have established that *D. cinerella* group includes *D. kasymovi* and probably *D. lavillei* whereas *D. zernyi* group includes *D. vaillanti* and *D. valentinae.* Highly supported phylogeny and results of species delimitation suggest the description of *D. elbrusica* **sp. nov.** and *D. sakartvella gidanica* **subsp. nov**. *Ps.* aff. *branickii* and *Ps.* aff. *nivosa* are new species based on DNA barcoding. The results of species delimitation show that genus *Pseudodiamesa* includes 10 (ASAP, GMYC), 14 (mPTP) or 21 (BOLD) distinct molecular taxonomic units (mOTUs) among which only *Ps. stackelbergi* have an undoubted species status that requires a large revision using both morphological and molecular approaches.

Key words: Diptera, Chironomidae, *Diamesinae, fauna,* taxonomy, new taxa, DNA barcoding, Caucasus

Introduction

According to the literature data based on the definition of adult males, 21 species of chironomids from the subfamily Diamesinae belonging to four genera are known for the Caucasus—*Boreoheptagyia legeri* (Goetghebuer), *Diamesa aberrata* Lundbeck*, D. bertrami* Edwards, *D. caucasica* Kownacki et Kownacka, *D. kasymovi* Kownacki et Kownacka, *D. latitarsis* (Goetghebuer), *D. lavillei* Serra-Tosio, *D. lindrothi* Goetghebuer, *D. martae* Kownacki et Kownacka, *D. modesta* Serra-Tosio*, D. sakartvella* Kownacki et Kownacka, *D. tonsa* (Haliday), *D. thomasi* Serra-Tosio, *D. tskomelidzei* Kownacki et Kownacka, *D. vaillanti* Serra-Tosio, *D. valentinae* Makarchenko, *Potthastia longimana* Kieffer*, P. gaedii* (Meigen)*, Pseudodiamesa* gr. *branickii , Ps. gorodkovi* Makarchenko and *Ps.* gr. *nivosa* (Kownacki 1980, 1985; Kownacki & Kownacka 1973a-b, 1974; Shilova 1978, 1988; Makarchenko 1983, 1990, 2022; Makarchenko *et al.* 2022). Most of these species are described or reported by authors from Georgia and Azerbaijan while about Diamesinae living in Armenia, North Ossetia, Dagestan, Kabardino-Balkaria and other regions, there was only information based on the identification of larvae in hydrobiological and other samples (Sinitsa 1934; Kasymov 1972; Kachvoryan *et al.* 2007; Khatukhov & Yakimov 2009; Petrova *et al*. 2011; Yakimov *et al.* 2014, 2015; Pezheva & Yakimov 2021 and other). The results of our study of Diamesinae adults collected by D.M. Palatov in 2012–2022 in various regions of the Caucasus, to some extent fill this gap.

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Below a list of all species Diamesinae found in some regions of the Caucasus in a tabular version.

In recent years, molecular-based approaches using fragment of cytochrome c oxidase I (COI) as DNA barcode were successfully adopted to delimit species of chironomids (Carew & Hoffmann 2015, Ekrem *et al.* 2010, Lin *et al.* 2018, Song *et al.* 2018) including species identification of Damesinae (Montagna *et al.* 2016, Lencioni *et al.* 2021). However, DNA barcodes for most Caucasian Diamesinae are missing.

The main aims of the present study were: (a) to make a morphological description with DNA barcoding of *Diamesa elbrusuca* **sp. nov.** and *D. sakartvella gidanica* **subsp. nov.** from the Elbrus Mount region; (b) to provide the DNA barcoding of Caucasian Diamesinae using COI sequences and calculate inter- and intraspecific genetic distances between obtained sequences and GenBank & BOLD system data; (c) in addition to the previous point to reconstruct a phylogeny and delimit species of *Diamesa* Meigen and *Pseudodiamesa* Goetghebuer for disentangle the taxonomical issues.

Materials and methods

The adults of chironomids were collected in Abkhazia (2012), North Ossetia (2018, 2020–2022), Dagestan (2021), Kabardino-Balkaria (2022) and preserved in 96% ethanol for DNA-analysis, in 70% ethanol for further study of morphology. The material was slide-mounted in polyvinyl lactophenol. The terminology follows Sæther (1980). The photographs were taken using an Axio Lab.A1 (Karl Zeiss) microscope with an AxioCam ERc5s digital camera, and then stacked using Helicon Focus software. The final illustrations were post-processed for contrast and brightness using Adobe® Photoshop® software.

Holotype and paratypes of the new species and subspecies, as well as all other material, are deposited in the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (FSCEATB FEB RAS).

Total genomic DNA was extracted from the thorax of adult mites or whole body of larvae using a Blood and Tissue Kit (Qiagen, Hilden, Germany) and the resultant DNA was eluted in 100 µl. The standard barcode region of the 5' end of the mitochondrial gene cytochrome c oxidase I (COI) was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994). The PCR reactions comprised a heating step at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s and elongation at 72°C for 1 min, with a final extension phase of 72°C for 5 min. PCR was performed in a reaction volume of 10 µl using 5 µl Go Taq Green Master Mix (Promega corp, Madison, WI, USA), 0.5 µM of each primer, 3 µl nuclease-free water, and 1.5 µl of genomic DNA. The PCR products were confirmed by electrophoresis in 1.5% agarose gels and then sequenced bidirectionally. Each PCR fragment was purified using Exonuclease I (ExoI) and Thermosensitive Alkaline Phosphatase (FastAP) (Thermo Fisher Scientific Inc., USA). Sequencing reactions had a total volume of 10 µl and included 10 pmol of each primer and reagents of BigDye terminator v3.1 cycle kit. The PCR products were bidirectional sequenced on an ABI 3130x sequencer (Applied Biosystems) and were aligned in MEGA7 (Kumar *et al.* 2016). Based on the observed p-distances are calculated inter- and intraspecific genetic distances also using MEGA7.

In addition to our own data, we used DNA barcodes of genus *Diamesa* from GenBank and BOLD systems which were close to Caucasian sequences (Fig. 19). Also, we used three sequences from each available BIN BOLD numbers of *Pseudodiamesa* (Fig. 20). Species delimitation for two genera was provided using distance-based approaches (BIN BOLD and ASAP) and tree-based approaches (mPTP and GMYC). Assemble Species by Automatic Partitioning (ASAP) analysis was implemented on the website (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html, Puillandre *et al.* 2021), with p-distances. Tree-based approaches Multi-rate Poisson tree processes (mPTP, Kapli *et al.* 2017) and general mixed Yule-coalescent (GMYC, Fujisawa & Barraclough 2013) were run on the web servers https://mptp.h-its.org/ and https://species.h-its.org/gmyc/ respectively using default parameters. The input ultrametric tree for GMYC was constructed using BEAST (Drummond *et al.* 2012). Settings were as follows: Strict clock, TN93+G nucleotide substitution model (Tamura-Nei, 93), Yule speciation process model (Gernhard 2008) and MCMC chain using 100 million generations.

Phylogenetic analysis was carried out on the DNA barcodes of *Diamesa* and *Pseudodiamesa* both sequenced data and mined from GenBank and BOLD systems. PartitionFinder 2.1.1 (Lanfear *et al.* 2012) is used to select the best-fit partitioning scheme and models separately for each codon position of protein coding genes using the greedy algorithm with linked branch lengths for the corrected Bayesian Information Criterion as the optimality criterion for model selection. The best models for the first, second and third codon position of COI was SYM+G (Zharkikh

1994), F81+I (Felsenstein 1981) and GTR+G (Tavare 1986) respectively for both trees. Bayesian phylogenetic analyses was carried out using Markov Chain Monte Carlo (MCMC) randomization in MrBayes v3.2.7 (Ronquist *et al*. 2012). Four Markov chains (three heated chains, one cold) were run for 5 million generations, with the first 25% of sampled trees discarded as burn-in. Strict clock model (brlenspr=clock:uniform) were used to obtain an ultrametric tree. Moreover, trace files of BI analysis were visually inspected in Tracer 1.7 (Rambaut *et al.* 2018) and then the tree is visualized in FigTree v. 1.4.4. The obtained sequences have been deposited in GenBank under numbers OQ282598-OQ282686 and OQ352248-OQ352261.

Fauna and taxonomy

TABLE 1. List of the Diamesinae of some Caucasian regions with distribution

Taxa	North Ossetia	Dagestan	Kabardino-Balkaria	Karachay-Cherkessia	Abkhazia	Azerbaijan	Georgia	**Distribution**
1	2	3	4	5	6	7	8	9
1. *Boreoheptagyia legeri* (Goetghebuer)	+	+	+		+	+	+	Palaeartic
2. *Diamesa aberrata* Lundbeck	+					+		Holarctic
3. *D. bertrami* Edwards	+	+	+		+			Holarctic
4. *D. caucasica* Kownacki et Kownacka	+					+	+	Endemic of Caucasus
5. *D. elbrusica* **sp. nov.**			+					Endemic of Caucasus
6. *D. hamaticornis* Kieffer	+				+		+	Palaearctic mountain
7. *D. kasymovi* Kownacki et Kownacka		+				+		Caucasus, Lebanon, Turkey
8. *D. latitarsis* (Goetghebuer)		+						Palaearctic
9. *D. lavillei* Serra-Tosio		+	+		+		+	Caucasus, Alps, Pyrenees
10. *D. lindrothi* Goetghebuer			+					Holarctic
11. *D. martae* Kownacki et Kownacka		+				+		Caucasus, Alps
12. *D. modesta* Serra-Tosio						+		West Palaearctic mountain
13. *D. parancysta* Serra-Tosio	+	+						Palaearctic
14. *D. sakartvella gidanica* **subsp. nov.**	+		+		+		+	Endemic of Caucasus
15. *D. tonsa* (Haliday)		+			+	+	+	West Palaearctic mountain
16. *D. thomasi* Serra-Tosio							+	Palaearctic and Oriental
17. *D. tskomelidzei* Kownacki et Kownacka							+	Endemic of Caucasus
18. *D. vaillanti* Serra-Tosio	+				+	+		Caucasus, Alps
19. *D. valentinae* Makarchenko	+		+	+			+	Endemic of Caucasus
20. *Potthastia longimana* Kieffer								Holarctic
21. *P. gaedii* (Meigen)								Holarctic
22. *Pseudodiamesa* gr. *branickii*	+	+	+		+		+	Holartic
23. *Ps. gorodkovi* Makarchenko				+				Endemic of Caucasus
24. *Ps.* gr. *nivosa*			+		+		+	Palaearctic
25. *Syndiamesa edwardsi* (Pagast)	+							West Palaearctic mountain
26. *S. nigra* Rossaro	+							Alps, Corsica, Caucasus
Total	12	9	8	2	9	8	11	

Notes. The following designation are accepted in the table: +, species present. Columns 2–6 show our data, columns 7–8 show data of Kownacki 1980, 1985; Kownacki & Kownacka 1973a-b, 1974; Shilova 1978; Makarchenko 1990 and our data.

As a result of the revision of adult males collected by D.M. Palatov in Abkhazia, North Ossetia, Dagestan and Kabardino-Balkaria, as well as available literature data on chironomids from Karachay-Cherkessia, Azerbaijan and Georgia, 26 species of the subfamily Diamesinae are registered for the Caucasus, belonging to 5 genera—*Boreoheptagyia* Brundin (1 species), *Diamesa* Meigen (18 species), *Potthastia* Kieffer (2 species), *Pseudodiamesa* Goetghebuer (3 species) and *Syndiamesa* Kieffer (2 species) (Table 1). Four species, *Diamesa hamaticornis*, *D. parancysta*, *Syndiamesa edwardsi* and *S. nigra*, are recorded for the first time for this region, one species, *D. elbrusica* **sp. nov.** and one subspecies *D. sakartvella gidanica* **subsp. nov.** are new to science and are described below. We classify 6 species as endemics of the Caucasus—*D. caucasica*, *D. elbrusica* **sp. nov.** *D. sakartvella sakartvella, D. sakartvella gidanica* **subsp. nov.***, D. tskomelidzei*, *D. valentinae* and *Ps. gorodkovi.* Five species with limited mountain distribution and apart from the Caucasus are known only in the Alps (*D. martae* and *D. vaillanti*), Lebanon and Turkey (*D. kasymovi*), the Alps and Pyrenees (*D. lavillei*) , Alps and Corsica (*S. nigra*). The areal of *D. thomasi* enters the Oriental region. Four species are distributed in the mountainous regions of the Western Palearctic, 6 in the Holarctic, and one is widely distributed in the Palaearctic (see Table 1). Unfortunately, we could not use the data given in the literature on larvae in the analysis of the fauna and distribution of Diamesinae from the Caucasus, since they must first be associated with adult males and clarified. For example, it is impossible to identify *D. insignipes* by the larva, which constitutes the *insignipes* group with at least 6 species and the larvae of which cannot be distinguished by morphology (Yakimov *et al.* 2015). Also, the identification by the larva and the record for Armenia of *D. tsutsuii* Tokunaga (Kachvoryan *et al.* 2007; Petrova *et al.* 2011) and for Kabardino-Balkaria *Pagastia orientalis* (Tshernovskij) (Pezheva & Yakimov 2021) are doubtful because the areals of these species do not extend beyond the Eastern Palaearctic.

For all the species known from the Caucasus, there are sufficiently detailed descriptions of adult males (Serra-Tosio 1971, 1972, 1976, 1989; Kownacki & Kownacka 1973a–b; Kownacki 1981; Rossaro 1980; Makarchenko 2022; Makarchenko *et al.* 2022), and we found it expedient not to redescribe them, but to make a descriptions only of *D. elbrusica* **sp. nov.** and *D. sakartvella gidanica* **subsp. nov.**

Descriptions

***Diamesa elbrusica* Makarchenko, Semenchenko et Palatov, sp. nov.**
http://zoobank.org/NomenclaturalActs/A0B111F2-DE17-42D2-931F-FB8FBDC1A1EC
(Figs 1–8)

Type material. Holotype, adult male, RUSSIA: Republic of Kabardino-Balkaria, Chereksky district, waterfall on the nameless river—the right tributary of the Mizhirga River, 1.3 km SE of the Bezengi alpine camp. It flows from the Gidan glacier and the Gidantau mountain, altitude 2410 m above sea level, 21.VII.2022, 43°06′16.92″N, 43°09′41.71″E, leg. D. Palatov. Paratypes: 5 adult males, the same data as holotype.

Derivatio nominis. The species is named as *elbrusica* after the type locality in the area of Elbrus Mount of the Central Caucasus.

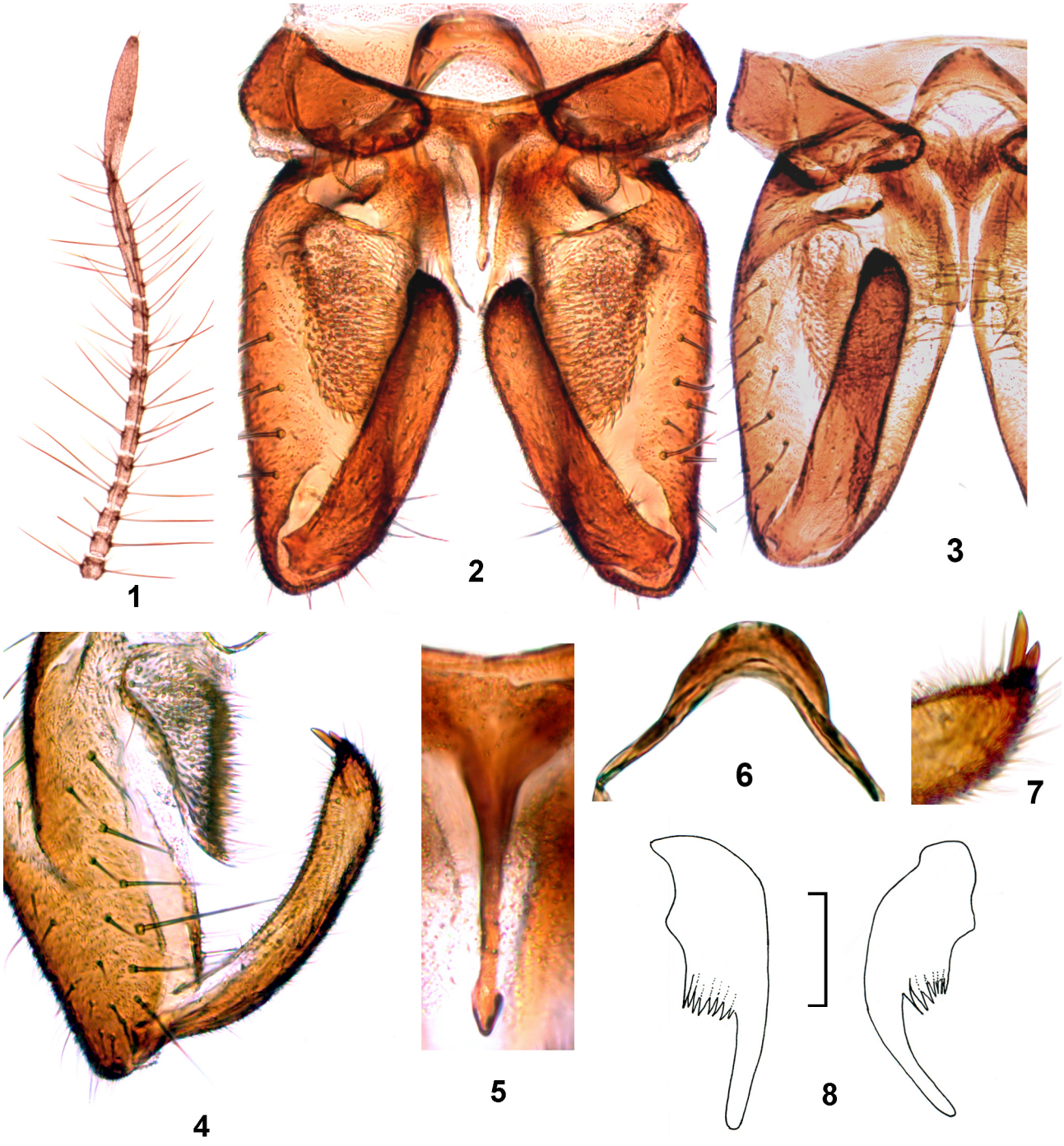
Description

Adult male (n = 4, except when otherwise stated). Total length 2.3–3.0 mm. Total length/wing length 1.02–1.12.

Coloration. Dark brown to black. Head, thorax and abdomen with hypopygium dark brown but gonostylus almost black. Legs light brown. Wings greyish.

Head. Eyes reniform, not hairy but pubescence by microtrichiae. Temporal setae including 6–7 preoculars, 5–7 verticals and 4 postorbitals. Clypeus with 8–12 setae. Antenna with 13 flagellomeres and reduced plume of setae (Fig. 1); length of these setae on flagellomeres 68–212 μm; terminal flagellomere with 1 subapical seta, 40–44 μm long; pedicel with 4 setae, 60–72 μm long. Length of 1–13 flagellomeres (μm): 68, 32–36, 28–36, 36, 30–40, 36, 36–40, 40–44, 44, 40–44, 44–52, 44–56, 360–364; AR 0.71–0.73. Palpomere length (μm): 44–48, 68–76, 104–116, 96–108, 100–128. Palpomere 3 in distal part with sensilla capitata with diameter 12 μm. Head width/palpal length 1.0–1.29.

Thorax. Antepronotum with 2–3 ventrolateral setae. Dorsocentrals 8–10, prealars 4–5. Scutellum with 13–14 setae in 2 rows.



FIGURES 1–8. Adult male of *Diamesa elbrusica* **sp. nov. 1,** flagellomeres 3–13 of antenna; **2–3,** hypopygium in dorsal view; **4,** gonocoxite and gonostylus in lateral view; **5,** anal point in dorsal view; **6,** transverse sternapodeme; **7,** apex of gonostylus; **8,** aedeagal lobes. Scale bar is 50 µm.

Wing. Length 2.44–2.68 mm, width 0.68–0.70 mm. Costal extension absent. Anal lobe rounded. Squama with 11–15 setae. R and R_1 with 21–23 setae, R_{4+5} with 2–3 setae in distal part. RM/MCu 2.5–2.8.

Legs. Spur of front tibia 52–60 µm long. Spurs of mid tibia 36–44 µm and 44–48 µm long. Spurs of hind tibia 64–72 µm and 36–44 µm long. Hind tibial comb with 13–14 setae. Length (µm) and proportions of leg segments are as in Table 2.

Hypopygium (Figs 2–8). Tergite IX narrow, with 8–13 setae from one side and with slender anal point, 96–100 µm long and 8–10 µm wide (Fig. 5), which slightly extended subapically. Laterosternite IX with 5–6 setae. Transverse sternopodeme (TSA) trapezoidal or rounded trapezoid (Figs 2, 6), 20–32 µm high, 92–140 µm wide; TSA

height/TSA width 0.19–0.33. Aedeagal lobe 116–132 μm long, weakly chitinized, wide in basal 2/3, with teeth in places along outer edge, narrow finger-like in distal third (Fig. 8); phallapodeme sclerotized, 96–108 μm long. Gonocoxite 280–300 μm long, on inner edge with long setae; inferior volsella 116–128 μm long and 76–80 μm wide, broad and flat, rounded apically, densely covered with microtrichia and setae (Figs 2–3); superior volsella reduced. Gonostylus 196–220 μm long and 44–48 μm wide, nearly straight and very long, subequal in width along entire length; gonostylus length/gonostylus width 4.33–4.78; apex with long macroseta, 20–22 μm long and tooth, 12–16 μm long; HR 1.31–1.44.

TABLE 2. Lengths (in μm) and proportions of leg segments of *Diamesa elbrusica* **sp. nov.**, male (n=4)

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5
P_1	1214–1353	1312–1476	820–968	377–492	262–279	82–98	115–131
P_2	1246–1460	1148–1345	508–623	279–344	172–197	82–98	98–131
P_3	1378–1525	1345–1574	836–1000	426–492	246–279	82–115	98–131

Continued.

	LR	BV	SV	BR
P_1	0.63–0.67	3.75–4.00	2.89–3.08	1.2–1.4
P_2	0.44–0.48	4.37–4.85	4.40–4.71	1.0–1.4
P_3	0.59–0.64	4.02–4.21	3.10–3.33	1.2–1.5

Pupa and ***larva*** unknown.

Diagnosis. Total length 2.3–3.0 mm. Eyes not hairy but pubescence by microtrichiae. Antenna with 13 flagellomeres and reduced plume of setae; AR 0.71–0.73. LR_1 0.63–0.67, BV_1 3.75–4.0, SV_1 2.89–3.08. Tergite IX narrow, with slender, fairly long anal point, which slightly extended subapically. Transverse sternopodeme trapezoidal or rounded trapezoid. Aedeagal lobe weakly chitinized, wide in basal 2/3 and with teeth in places along outer edge, narrow finger-like in distal third. Inferior volsella broad and flat, rounded apically, densely covered with microtrichia and setae. Gonostylus nearly straight and very long, subequal in width along entire length; apex with long macroseta, and tooth; HR 1.31–1.44. *D. elbrusica* **sp. nov.** is closely related to *D. arctica* (Boheman) and *D. spinacies* Sæther but males of both species are larger (4.5–6.3 mm), with plumose antenna, AR > 1, aedeagal lobes with another structure and gonostylus has different shape; HR 1.64–1.98.

Ecology. Adult males were collected from stones and boulders in mountain stream below a multi-meter waterfall, located at an altitude of 2410 m a.s.l., at a flow rate of 0.7–1 m/s, with water temperatures 7°C.

Distribution. Known only from the type locality in Elbrus Mount area of the Central Caucasus (Figs. 17–18).

Diamesa sakartvella sakartvella Kownacki et Kownacka
(Figs. 9–11)

Diamesa sakartvella Kownacki et Kownacka, 1973: 21; Langton & Visser 2003: 43; Ashe & O'Connor 2009: 284; Makarchenko *et al.* 2022: 501.

Remarks. In comparing the results of DNA barcoding of adult males of *Diamesa sakartvella* Kownacki et Kownacka, 1973 from Republic of Kabardino-Balkaria (Russia) and Republic of North Ossetia-Alania (Russia), it turned out that the p-distance obtained using the site of cytochrome oxidase I mtDNA 658 bp long between the populations is 4.16%. The results of species delimitation using BOLD, ASAP, mPTP and GMYC suggest relation this populations to different species. However, we did not reveal sufficient morphological differences of these chironomids to establish independent species and found it appropriate to give subspecies status for these populations, namely *D. sakartvella sakartvella* for specimens from Georgia and North Ossetia, and *D. sakartvella gidanica* **subsp. nov.** for specimens from Elbrus Mount area of Kabardino-Balkaria.

For *D. sakartvella sakartvella*, a detailed description of the adult male is already available (Kownacki & Kownacka 1973; Makarchenko *et al.* 2022), and we decided not to redescribe it but only compare below with the *D. sakartvella gidanica* **subsp. nov.** However, when reading the description in paper of Makarchenko *et al.* (2022), it

should be borne in mind that when preparing the manuscript, the senior author made a mistake in indicating superior volsella instead of inferior volsella.

Distribution. This subspecies is known from Georgia and North Ossetia of Caucasus.

***Diamesa sakartvella gidanica* Makarchenko, Semenchenko et Palatov, subsp. nov.**
http://zoobank.org/NomenclaturalActs/374B4F78-BC42-4EEB-9776-34EFC914E877
(Figs 12–16)

Type material. Holotype, adult male, RUSSIA: Republic of Kabardino-Balkaria, Chereksky district, waterfall on the nameless river—the right tributary of the Mizhirga River, 1.3 km SE of the Bezengi alpine camp. It flows from the Gidan glacier and the Gidantau mountain, altitude 2410 m above sea level, 21.VII.2022, 43°06′16.92″N, 43°09′41.71″E, leg. D. Palatov. Paratypes: 6 adult males, the same data as holotype.

Derivatio nominis. The subspecies is named as *gidanica* after the type locality near Gidan glacier.

Description

Adult male (n = 4, except when otherwise stated). Total length 2.1–2.2 mm. Total length/wing length 0.92–0.97.

Coloration. Brown to dark brown. Head, thorax and abdomen dark brown. Legs brown. Wings gray, with brownish veins.

Head. Eyes hairy, reniform. Temporal setae including 2–4 frontals, 3–7 orbitals, 5–13 verticals. Frontal tubercles 8–12 μm height and *ca* 60 μm width. Clypeus with 4–7 setae. Antenna with 8 flagellomeres and reduced plume of setae; number and length of these setae on 1–7 flagellomeres respectively: 1–2 (40 μm), 3 (52–60 μm), 2 (52 μm), 1 (48 μm), 2 (52–60 μm), 0, 0; terminal flagellomere with 5 setae, 60–80 μm long in basal part and with 2 subapical setae, 20–28 μm long. Length of 1–8 flagellomeres (μm): 72–84, 32–44, 36–40, 24–28, 24–28, 24, 24, 140–164; AR 0.61–0.66. Palpomere length (μm): 32–44, 64–72, 10–112, 84–88, 84–96. Palpomere 3 in distal part with sensilla capitata with diameter 28 μm. Head width/palpal length 0.93–1.06. Antennal length/palpal length 1.0–1.1.

Thorax. Antepronotum with 7–9 ventrolateral setae. Dorsocentrals 10–11, 90–100 μm long; prealars 3, 92–104 μm long. Scutellum with 8–10 setae in 1 row.

Wing. Length 2.1–2.4 mm, width 0.60–0.68 mm. Anal lobe reduced. Squama with 6–15 setae, 40–56 μm long. R and R_1 with 17–20 setae, R_{4+5} with 6 setae in distal half. RM/MCu 2.7–3.5.

Legs. Spur of front tibia 32 μm long. Spurs of mid tibia equal 32 μm long and 36 μm long. Spurs of hind tibia 52–56 μm and 32 μm long. Hind tibial comb with 18–21 setae. Length (μm) and proportions of leg segments are as in Table 3.

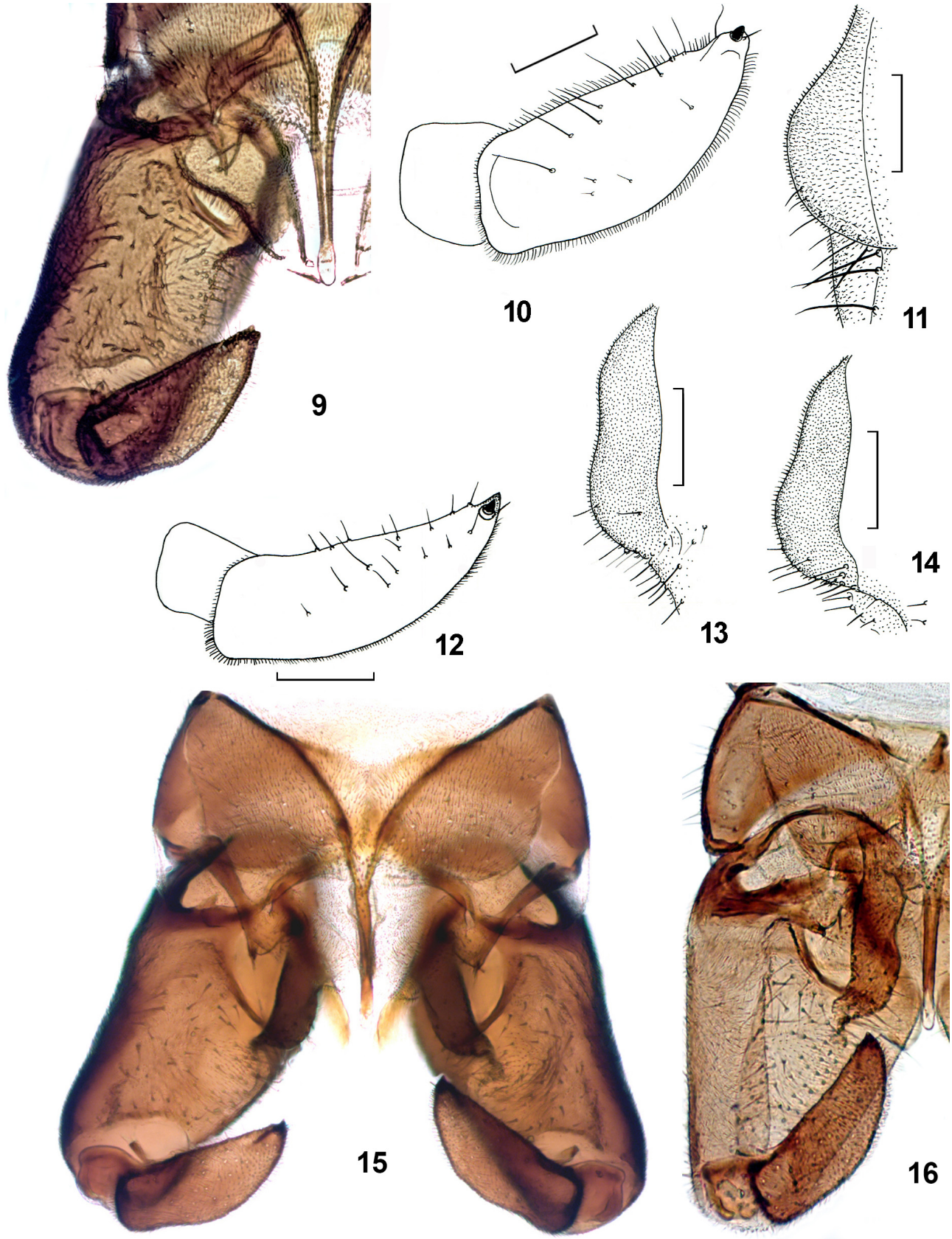
TABLE 3. Lengths (in μm) and proportions of leg segments of *Diamesa sakartvella gidanica* **subsp. nov.**, male (n=4)

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5
P_1	1394–1607	1337–1509	853–1000	394–476	246–295	82–98	107–131
P_2	1394–1558	1148–1361	508–574	271–295	164–180	82	98
P_3	1443–1640	1345–1574	853–1000	459–525	246–279	82–115	115–131

Continued.

	LR	BV	SV	BR
P_1	0.63–0.65	4.07–4.28	3.20–3.28	1.4–1.6
P_2	0.42–0.44	4.93–5.33	4.49–5.09	1.4–1.6
P_3	0.63–0.64	3.94–4.21	2.94–3.27	1.6–1.8

Hypopygium (Figs 12–16). Tergite IX densely covered with strong macrotrichia apices of which are directed anteriorly, with 9–12 setae and with narrow (14–16 μm), chitinized and naked anal point, 196–220 μm long (Figs 15–16). Laterosternite IX with 5–7 setae, 18–21 μm long. Transverse sternopodeme (TSA) wide triangular, 48–76 μm high, 152 μm wide at the base; TSA height/TSA width 0.32–0.50. Phallapodeme sclerotized, 84 μm long (n=1). Gonocoxite 280–332 μm long; inferior volsellae rounded and elongated, with some setae in basal part (Figs. 13–14). Gonostylus 160–180 μm long and 52–56 μm wide; in apical part with megaseta in form of wide terminal spine, *ca* 12 μm long and tooth the same size, next to it there is two setae approximately of the same length (Figs.12, 15–16). HR 1.75–1.93.



FIGURES 9–16. Adult males of *Diamesa sakartvella sakartvella* Kownacki et Kownacka **(9–11)** and *D. sakartvella gidanica* **supsp. nov. (12–16). 9, 15–16,** hypopygium in dorsal view; **10, 12,** gonostylus; **11, 13–14,** inferior volsellae. Scale bars are 50 μm.

Diagnosis. As noted above, the main differences between the two subspecies were obtained by DNA barcoding of adult males from North Ossetia and Kabardino-Balkaria, and this will be discussed in more detail in chapter "Results of DNA barcoding". Here we tried to give some morphological differences, which are that *D. sakartvella gidanica* **subsp. nov.** males have a smaller total length (2.1–2.2 mm), shorter wings (2.1–2.4 mm) and legs, different TL/WL value (0.92–0.97); number of temporal frontal setae 2–4, AR 0.61–0.66, clypeals 4–7, scutellars 8–10, LR_2 0.42–0.44, BV_2 4.93–5.33, SV_2 4.49–5.09, inferior volsellae rounded and elongated (Figs. 13–14), HR 1.75–1.93. Total length of *D. sakartvella sakartvella* 2.9–3.1 mm, wing length 2.4–2.8 mm, TL/WL 1.04–1.19, number of temporal frontal setae 7–8, AR 0.58, clypeals 9, scutellars 10–12, LR_2 0.45–0.49, BV_2 4.35–4.98, SV_2 4.56–4.69, inferior volsellae rounded and not elongated (Fig. 11), HR 2.02–2.10. Also, in males of these subspecies, the shape of the gonostylus is slightly different (Figs 9–10, 12, 15–16).

Ecology. Adult males were collected together in the same biotope with *D. elbrusica* **sp. nov.**, from stones and boulders in mountain stream below a multi-meter waterfall, located at an altitude of 2410 m a.s.l., at a flow rate of 0.7–1 m/s, with water temperatures 7°C.

Distribution. Known only from the type locality in Elbrus Mount area of the Central Caucasus (Figs. 17–18).

FIGURES 17–18. Locality of *Diamesa elbrusica* **sp. nov.** and *D. sakartvella gidanica* **subsp. nov.** RUSSIA: Republic of Kabardino-Balkaria, Chereksky district, waterfall on the nameless river—the right tributary of the Mizhirga River, 1.3 km SE of the Bezengi alpine camp (Photos by D.M. Palatov).

Results of DNA barcoding

In the present study, we used the COI DNA barcode for comparison with previously published sequences of non-biting midges from Caucasus (Makarchenko *et al.* 2022 a-b) and for disentangle the taxonomical issues of *Diamesa* and *Pseudodiamesa*. To achieve this, we calculated genetic p-distance values at intra- and intraspecific levels, reconstructed Bayesian phylogenetic trees and used approaches for single locus species delimitation such as distance-based Barcode Index Number (BIN), Assemble *Species* by Automatic Partitioning (ASAP) and tree-based multi-rate Poisson tree process (mPTP), generalized mixed Yule coalescent (GMYC). We calculated only genetic distances for *Boreoheptagyia* and *Syndiamesa* as these sequences were new for GenBank.

Overall, we have sequenced fragments of the cytochrome oxidase subunit I (560–658 bp in length) of 102 of Diamesinae from four genera: *Boreoheptagyia* Brundin, *Diamesa*, *Pseudodiamesa* and *Syndiamesa* Kieffer. Of these, 58 sequences belong to 15 species and 2 subspecies of genus *Diamesa*. Another 23 sequences belonged to *Ps.* aff. *branickii* and 6 sequences to *Ps.* aff. *nivosa.* The remaining 12 sequences belonged to *B. legeri*, 2 sequences to *S. nigra* and 1 to *S. edwardsi*.

In previous years, we sequenced chironomids larvae morphologically close to *B. legeri* (OQ282627-OQ282629, OQ282641-OQ282642, OQ282662-OQ282664). However, in 2022, an adult male (EAM1285, OQ352261) was collected, the results of DNA barcode and morphology analysis confirmed the correctness of the larvae identification. The average intraspecific pairwise p-distance for 12 samples of *B. legeri* was 0.68%. The three closest species from GenBank were *B. brevitarsis* Tokunaga (MT240772-MT240776, BOLD:AEG1498) from Iran, *Boreoheptagyia* sp. (KY640386, BOLD:ADV5485) from Kyrgyzstan and *B. kurobebrevis* (MZ128907-MZ128908, MZ043576, BOLD: AEE4515) from China. The average interspecific distances between *B. legeri* and three species were 9.16%, 11.26% and 11.84% respectively. *B. brevitarsis* was collected geographically closest to the Caucasus, which, coupled with low genetic distances, may indicate their monophyly.

We obtained the partial COI sequences of 58 *Diamesa* specimens, of which 10 specimens were *D. vaillanti*, 8—*D. tonsa*, 5—*D. bertrami*, *D. caucasica*, *D. hamaticornis* and *D. kasymovi*, 4—*D. lavillei*, 3—*D. valentinae* and *D. elbrusica* **sp. nov.**, 2—*D. latitarsis*, *D. lindrothi*, *D. martae* and *D. sakartvella gidanica* **subsp. nov.**, 1—*D. aberrata* and *D. thomasi* (Fig. 19). Pairwise intraspecific sequence divergence for most species did not exceed 1%. The exception was *D. bertami*, *D. kasymovi*, *D. vaillanti* and *D. valentinae* with intraspecific distances 1.42%, 1.10%, 1.08% and 1.07% respectively.

Phylogenetic tree was reconstruct using obtained sequences of *Diamesa* as well as DNA barcodes of Caucasian *Diamesa* (both marked in blue) and non-Caucasian sequences of closest samples from GenBank and BOLD systems. The aim of this tree is to show the relationships of the Caucasian *Diamesa*, and to discuss some taxonomic issues.

D. thomasi was the earliest branching lineage with high support (Bayesian Posterior Probability, BPP=1). The next polytomic clade was well supported (BPP=0.94) and includes the remaining *Diamesa* samples. The sequences of *D. bertrami* from Caucasian were close to *Diamesa bertrami* from Alps (LN897625-LN897627) except specimen EAM1039 (OQ282644) which formed an independent clade (BPP=1.00). Genetic divergence between EAM1039 and sequences of the remaining clade was 2.82% which is more likely to be the intraspecific level (Montagna et al., 2016) despite the results of mPTP and GMYC analyses (Fig. 19). Specimen EAM1039 was collected in the same locality with specimen EAM1038 (OQ282643) and about 230 kilometers from the other Caucasian *D. bertrami* specimens that suggest the existence of two sympatric populations of the same species.

D. aberrata and *D. sakartvella* was formed a common moderately supported clade (BPP=0.86). The results of species delimitation indicate that *D. aberrata* from Caucasian conspecific to *D. aberrata* from Austria, Germany, Greenland and Norway (BIN BOLD:AAB1737). *D. sakartvella* divided into two clades, formed by specimens from the Republic of North Ossetia-Alania (OM867255-OM867259) and Republic of Kabardino-Balkaria (OQ282679, OQ282686). High average genetic distances between groups (4.16%), strongly supported monophyly (BPP=1.00), results of four analysis of species delimitation (BOLD, ASAP, mPTP and GMYC) and morphological differences (see above) are the reasons to delimit *D. sakartvella* into two subspecies: *D. sakartvella sakartvella* and *D. sakartvella gidanica* **subsp. nov.**

The average genetic distances between *D. lindrothi* from Caucasian and Norway (not shown on Fig. 19) were 4.05% and included into distinct BIN BOLD numbers (BIN BOLD:AFA7447 and BOLD:AAC4741 respectively) which suggest their non-conspecificity. COI sequences of *D. martae* (OQ282617, OQ282653) were obtained for the first time.

D. latitarsis formed two sister clades with minor genetic distance (1.6%) although mPTP analysis suggests that they are different species. Caucasian sequence of *D. latitarsis* are part of BIN BOLD:AAC7191 and expand the coverage of sequences in addition to Iceland, Italy, Germany and Norway.

D. elbrusica **sp. nov.** and *D. caucasica* formed two sister clades with high support (BPP=1.00) and interspecific genetic distances equal to 4.75%. Four analyses for species delimitation confirmed species independence of *D. elbrusica* **sp. nov.** and *D. caucasica*. Thus, here we make a description of the *D. elbrusica* **sp. nov**. Recently described species *Diamesa caucasica* (Makarchenko *et al.* 2022) has been supplemented by five new sequences although all collections are limited by Republic of North Ossetia–Alania (Russia).

The latest branches include two clades, each containing many *Diamesa* species. The first clade containing *Diamesa vaillanti, D. valentinae* from Caucasius as well as *D. bohemani* and *D. zernyi* Europe (GenBank data). The remaining clade includes *D. hamaticornis, D. kasymovi, D. laviellei, D. tonsa* and *D. cinerella, D. hyperborea* Europe (GenBank data). Four approaches for species delimitation showed that *D. vaillanti, D. valentinae, D. bohemani* and *D. zernyi* belong to the same species. In turn tree-based approaches for species delimitation (mPTP and GMYC) showed that *D. laviellei* belongs to a unique mOTU.

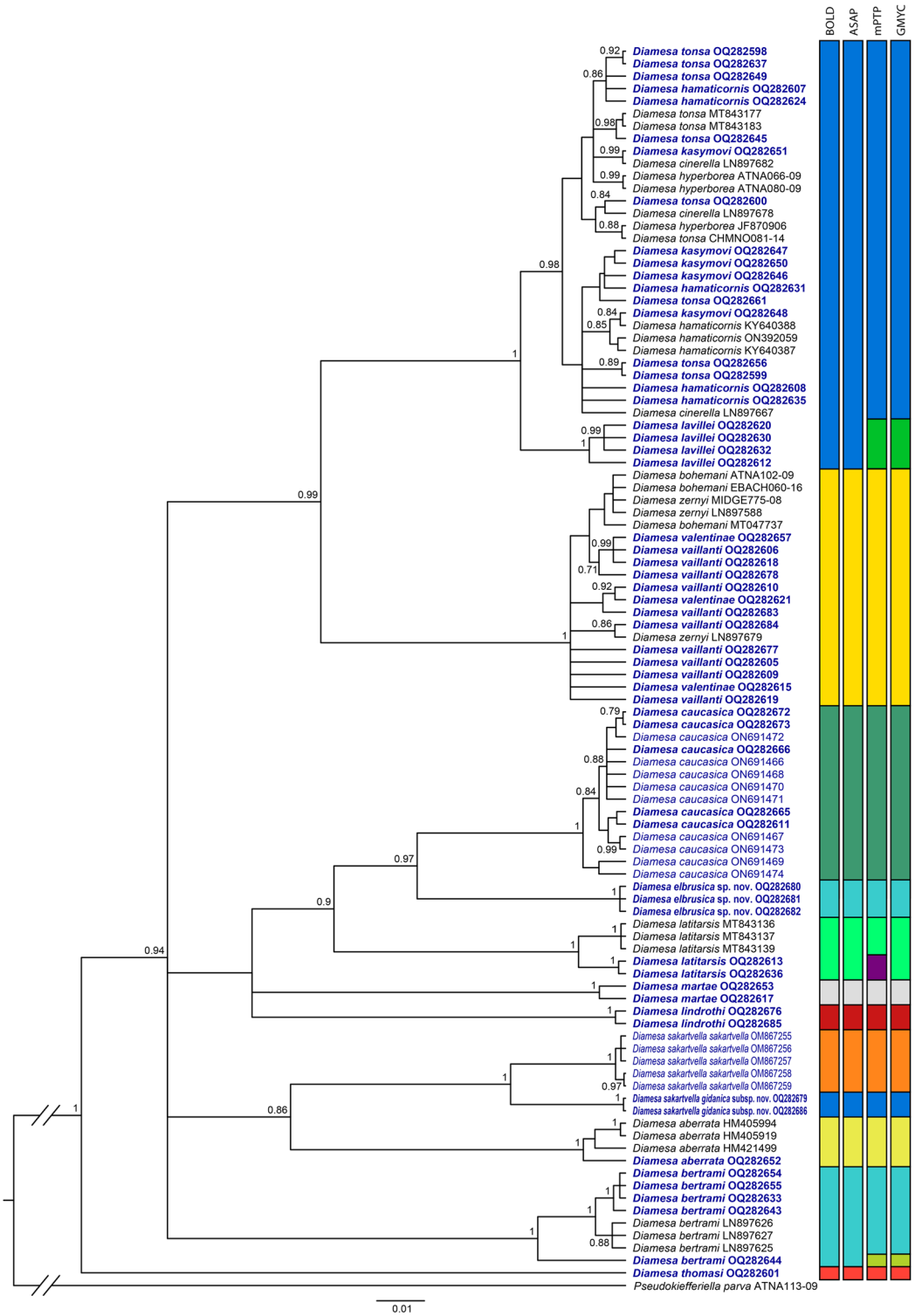


FIGURE 19. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Diamesa* Meigen and outgroup *Pseudokiefferiella parva* (Edwards). Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens from Caucasian are in blue while obtained sequences in this study are in bold.

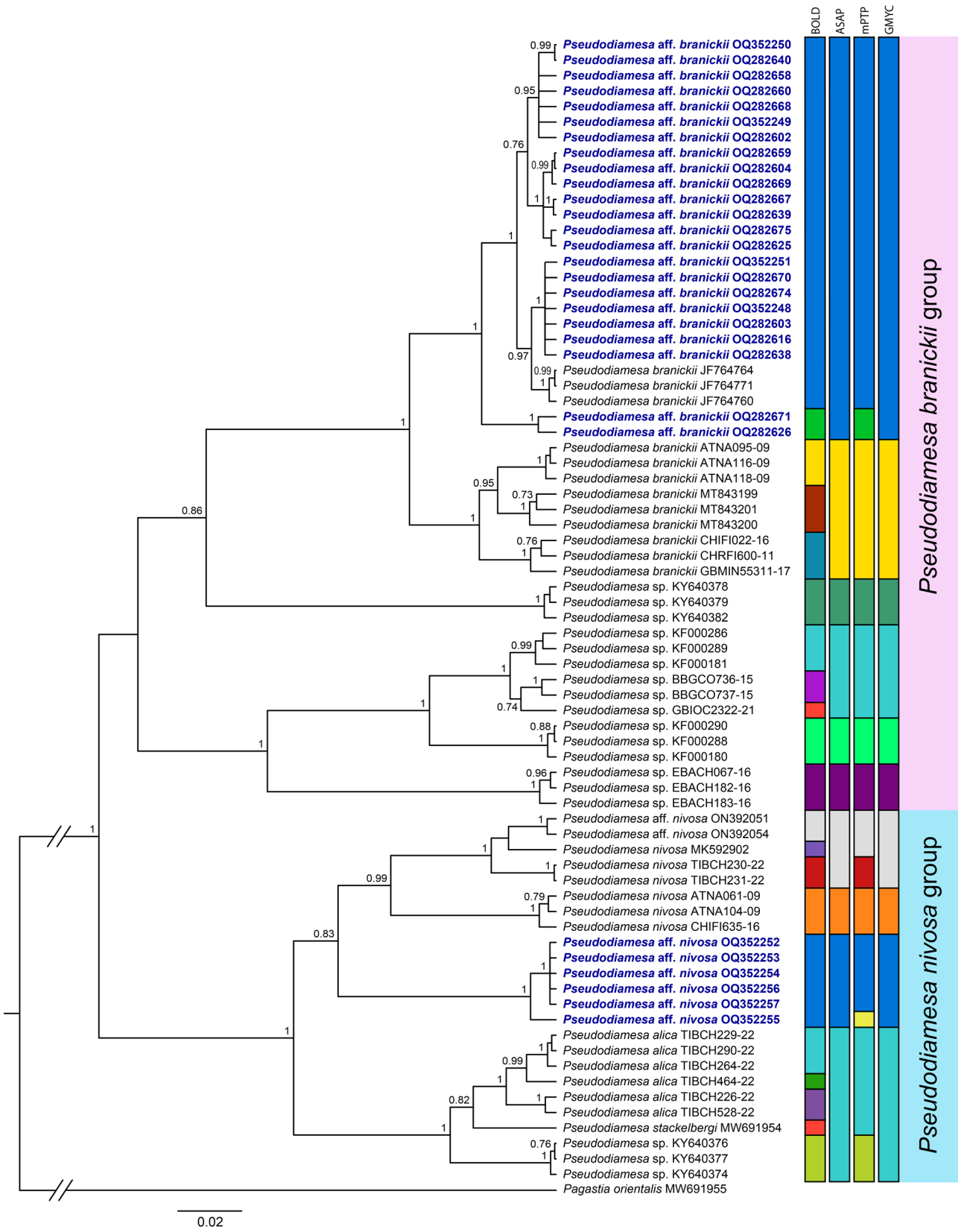


FIGURE 20. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Pseudodiamesa* Goetghebuer and outgroup *Pagastia orientalis* (Chernovsky). Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens from Caucasian are in blue while obtained sequences in this study are in bold.

Recent work has shown that *D. cinerella, D. tonsa*, and *D. hamaticornis* on one hand, and *D. bohemani* and *D. zernyi* on the other one has extremely low interspecific distances. Pairwise p-distances between *D. cinerella*, *D. tonsa* was 0–0.040 (Lencioni *et al.* 2021) or 0.9% in average using K2P distances (Montagna *et al.* 2016). Similar values for the *D. bohemani* and *D. zernyi* were 0–0.020 (Lencioni *et al*. 2021). The high similarity of these groups of species was confirmed by ABGD analysis, median-joining network analysis as well as due to the paraphyly in phylogenetic reconstructions using both only COI sequences and multilocus approach (mitochondrial COI, COII, and the nuclear gene 18S rRNA). In addition, the authors showed that the head capsule color is not a good taxonomic character to differentiate the larva of *D. zernyi* and *D. cinerella* because it is subject to phenotypic plasticity, however, these and other species have a unique morphological characters of the male genitalia. The authors came to the conclusion that *D. bohemani–D. zernyi* and *D. cinerella–D. tonsa* are populations of the same species that diverged due to climatic events during the glacial period in the Pleistocene (Lencioni *et al*., 2021). Ekrem *et al*. (2010) also suggest that *D. bohemani* and *D. zernyi* should be regarded as synonymous.

Here we have expanded each group with new species. To the group *D. cinerella*, *D. tonsa*, and *D. hamaticornis* we have added obtained *D. kasymovi* and also *D. hyperborea* from the GeneBank and BOLD Systems. *D. laviellei* was placed as sister to the clade uniting *D. cinerella*, *D. hamaticornis, D. hyperborean, D. kasymovi* and *D. tonsa* (BPP=1.00), however, interspecific distances between them were low (2.74%, 2.67%, 2.58%, 3.07%, 2.84% respectively). The three methods suggest that *D. laviellei* is an independent species, while ASAP combined 6 species into one species. Group *D. bohemani–D. zernyi* was supplements with *D. vaillanti* and *D. valentinae.* The average p-distances within group *D. bohemani–D. vaillanti–D. valentinae–D. zernyi* were 0.92% (0.44–0.98%). Four methods for species delimitation assume conspecifity of all species.

The problem of genetic homogeneous of revealed groups should be resolved by additional studies, including the reconstruction of the phylogenetic relationships of most species of the genus *Diamesa* using multilocus approaches, as well as the use of NGS data for species identification. The range of both groups has expanded significantly and the number of species has increased, making it difficult to explain the observed results by glacial processes or low morphological differences. Therefore, we agree with Montagna *et al*. (2016) that the main reason for the high similarity of species within groups is incomplete lineage sorting.

Chironomid larvae of the genus *Pseudodiamesa* were divided into two groups, *Ps.* gr. *branickii* and *Ps.* gr. *nivosa* (Makarchenko & Makarchenko 1999). The first group includes *Ps. branickii* Nowicki and *Ps. pertinax* Garrett and the second consist of *Ps. arctica* Malloch, *Ps. gorodkovi* Makarchenko, *Ps. latistyla* Makarchenko, *Ps. mongolzecea* Sasa & Suzuki, *Ps. nepalensis* Reiss, *Ps. nivosa* Goetghebuer, *Ps. stackelbergi* Goetghebuer, *Ps. subnivosa* Linevich & Makarchenko, *Ps. sunabacedea* Tanaka & Sasa*, Ps. vetusta* Makarchenko (Ashe & Connor 2009, Ilyashuk *et al.* 2010). We obtained sequences for two species—*Ps.* aff. *branickii* and *Ps.* aff. *nivosa*, which are currently defined to a group level. The average intraspecific pairwise p-distances of two species were 1.68% and 0.35% respectively. For the *Ps.* aff. *nivosa*, our material contained only larval, while for *Ps.* aff. *branickii* six specimens (OQ282616, OQ282638-OQ282640, OQ282671, OQ352251) were imago males what is the basis for its description in the future. We reconstruct a phylogenetic tree to compare the obtained sequences with the other *Pseudodiamesa* DNA barcodes and raise questions of the taxonomic situation of the genus. The Bayesian Inference revealed two well-supported primary clades (BPP=1), one including *Ps. nivosa* and *Ps.* aff. *nivosa, Ps. stackelbergi, Ps. alica* (questionable synonym of *Ps. nivosa,* Ashe & Connor 2009) and one including *Ps. branickii, Ps.* aff. *branickii* or species without a group level. We assume that the revealed clades correspond to *Ps. branickii* and *Ps. nivosa* groups. *Ps.* aff. *nivosa* from Caucasian was placed in the clade with members of *Ps. nivosa* and *Ps.* aff. *nivosa* (BPP=0.83). The results of species delimitation assume that *Ps.* aff. *nivosa* from Caucasian is a new mOTU. The sister well supported clade (BPP=1) consists of *Ps. alica* from China, *Ps. stackelbergi* from Russia and *Pseudodiamesa* sp. (KY640374, KY640376, KY640377) from Tajikistan. *Ps. stackelbergi* was placed to the separate mOTU only in accordance with BIN BOLD data while ASAP and GMYC suggest that all DNA barcodes belongs to single mOTU. Caucasian *Ps.* aff. *branickii* and *Ps. branickii* (JF764760, JF764764, JF764771) from Iran (BIN BOLD:ACD6211) belongs to single mOTU according to four methods for species delimitation although two specimens, EAM386 and EAM1197 (OQ282626 and OQ282671) belong to unique mOTU using BOLD (BIN BOLD: AFA2841) and mPTP. Highly supported (BPP=1) sister clade consist *Ps. branickii* from Europe witch divided into tree BIN BOLD numbers (BOLD:AAD0382, BOLD:ACX2423, BOLD:AEE1136). In addition, six BIN BOLD numbers of *Pseudodiamesa* have been deposed in the BOLD systems and GenBank, which probably belongs to *Ps. branickii* group. Thus, to date genus *Pseudodiamesa* has 10 (ASAP, GMYC), 14 (mPTP) or 21 (BOLD) distinct

mOTU among which the «true» *Ps. branickii* and *Ps. nivosa* are unknown, *Ps. alica* which is probably belong to a distinct species and *Ps. stackelbergi* which is beyond doubt. The genus *Pseudodiamesa* requires a large revision using both morphological and molecular approaches.

DNA barcodes for *S. edwardsi* and *S. nigra* were first obtained and deposed in the GeneBank and BOLD systems. Intraspecific distance between two *S. nigra* specimens was 0.91%. The interspecific distance between two species was 6.99% that correspond to species level.

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